HAX-1, Identified by Differential Display Reverse Transcription Polymerase Chain Reaction, Is Overexpressed in Lesional Psoriasis

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Psoriasis is a chronic inflammatory disease characterized by epidermal hyperplasia and an inflammatory infiltrate. The normal differentiation from basal to granular keratinocytes with subsequent apoptosis and cornification is disturbed in the akanthotic epidermis. This could be due to both an excess of mitogenic stimuli with hyperproliferation and/or resistance to apoptosis. By mRNA differential display we found HAX-1 to be overexpressed in lesional psoriatic skin. The overexpression of HAX-1 was verified at the mRNA level by Northern blot and *in situ* hybridization, as well as at the protein level by Western blot and immunohistochemistry. Detection of HAX-1 in mRNA from different tissues showed strong expression in the brain, pancreas, skeletal muscle, and heart. In contrast to primary keratinocytes and melanocytes we found HAX-1 highly expressed in human immortalized keratinocytes (HaCaT) and different melanoma cell lines. In HaCaT cells as a model for psoriatic keratinocytes we found an increased ultraviolet-induced apoptosis after expression of HAX-1 antisense mRNA. In psoriasis, the epidermal differentiation could be disturbed due to the increased expression of HAX-1 and hence a prolonged resistance to terminal differentiation. Antiapoptotic mechanisms are an emerging concept for the understanding of the pathogenesis of this disease possibly leading to clinical applications.
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Psoriasis is a chronic, inflammatory, hyperproliferative disease affecting about 2% of the Caucasian population. A large number of experimental studies indicate that psoriasis is caused by a combination of genetic, immunologic and environmental factors (for review, see Christophers, 1996; Nickoloff, 1999; Elder *et al*, 2001). The association of psoriasis and HLA types was shown in case-control studies, leading to the identification of HLA-Cw6 and HLA-B57 as major psoriasis-associated HLA types (Elder *et al*, 1994). A number of environmental factors, e.g., stress and streptococcal infection, as well as specific antigens from retroviruses, bacterial superantigens, or homologous endogenous proteins were also shown to be involved in psoriasis manifestation (for review, see Nickoloff, 1999).

The pathogenesis of psoriasis involves (i) the infiltration and activation of immune cells (Bata-Csorgo *et al*, 1995; Wrone-Smith and Nickoloff, 1996) and (ii) differences in keratinocyte growth and differentiation (McKay and Leigh, 1995). Quite recently it was shown that psoriasis can be induced by transfer of cells harboring natural killer receptors and might therefore result from a cutaneous defect, which is triggered by an autoimmune activation (Gilhar *et al*, 2002). In psoriatic skin the local immunologic state is characterized by a bias towards a Th1-specific immune response and the secretion of the respective cytokines (Uyemura *et al*, 1993).

A number of studies led to the hypothesis that the strong epidermal thickening (akanthosis) within psoriatic plaques might be caused by disturbed apoptosis in addition to the observed hyperproliferation. It was shown that psoriatic keratinocytes exhibit an abnormal resistance to apoptosis (Wrone-Smith *et al*, 1995; 1997). One of the first proteins discussed to be involved in apoptosis resistance of keratinocytes was Bcl-x (Wrone-Smith *et al*, 1995). As Bcl-x-overexpressing mice do not show epidermal features similar to psoriasis, however, additional factors seem to be involved. More recently, interleukin-15 was shown to be expressed in keratinocytes from psoriatic lesions and its role in inhibition of apoptosis was analyzed (Rückert *et al*, 2000). Expression of either protein, Bcl-x or interleukin-15, was shown to inhibit Fas-induced apoptosis. Interestingly, members of the ov-serpin family, e.g., hurpin (Abts *et al*, 1999) and SCCA1 (Duk *et al*, 1989), were also shown to be overexpressed in psoriatic skin. An antiapoptotic function has been suggested for both proteins. It can be speculated that additional genes are involved in the disturbed regulation of apoptosis in psoriatic tissue.

To identify such disease-associated genes, a number of techniques have been established and applied to psoriatic tissue. In contrast to DNA-based strategies, RNA-based techniques were

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Abbreviation: DDRT-PCR, differential display of reverse-transcribed mRNA polymerase chain reaction.

shown to be more applicable to the isolation of psoriasis-specific genes. By differential display of reverse-transcribed mRNA polymerase chain reaction (DDRT-PCR) (Liang and Pardee, 1992) mRNA molecules are amplified using arbitrary PCR primers and subsequently visualized by gel electrophoresis. A comparison between two RNA populations, e.g., from psoriatic lesional and nonlesional skin, allows the identification of differentially expressed genes. Isolated PCR fragments can subsequently be subcloned and sequenced.

By using DDRT-PCR we have searched for differentially expressed genes in psoriatic skin by comparing RNA isolated from lesional and nonlesional skin. Expression of the apoptosis-related gene HAX-1 was further verified by Northern and Western blots using skin biopsies, different melanoma cell lines, HaCaT cells, and keratinocytes. We further studied the antiapoptotic activity of HAX-1 in HaCaT cells, which exhibit proliferative activity and apoptosis resistance similar to psoriatic keratinocytes. A possible role of HAX-1 in the process of apoptosis regulation in psoriasis is discussed.

MATERIALS AND METHODS

Skin samples Split-thickness sections (0.3 mm), which were predominantly composed of epidermis, were obtained from the backs of patients with untreated acute plaque-type psoriasis (Schulz *et al*, 1993). For control purposes, unaffected skin specimens were obtained from clinically normal skin areas. Normal skin was donated by healthy volunteers. All biopsies were taken with the informed patient's consent and approval of the local ethical committee.

Cell culture The spontaneously transformed human epidermal cell line HaCaT and the human melanoma cell lines MV3, BLM, and SK-Mel-28 were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Eggenstein, Germany) supplemented with 10% fetal bovine serum, 100 U per μl penicillin, and 100 pg per μl streptomycin. Normal human keratinocytes were obtained by dispase treatment of breast skin specimens (15×10 cm) and cultured under serum-free conditions in keratinocyte basal medium (Biowhittaker, Vervier, Belgium). Primary human melanocytes were obtained from PromoCell (Heidelberg, Germany) and cultured in phorbol-myristate-acetate-free medium (PromoCell). Cell culture was maintained in a 37°C incubator in a moist atmosphere of 5% CO_2.

RNA isolation Shave biopsies were snap frozen in liquid nitrogen. Biopsies were homogenized using a dismembrator (Braun-Melsungen, Melsungen, Germany). The powder was immediately transferred into Trizol™ reagent and total RNA was isolated according to the manufacturer's instructions (Gibco BRL). Contaminating DNA was removed by treatment with DNase I (Boehringer Mannheim, Germany). RNA quality was controlled by agarose gel electrophoresis.

DDRT-PCR The DDRT-PCR technology was first described by Liang and Pardee (1992). The protocol used has been successfully employed for the identification of ultraviolet (UV) regulated genes (Abts *et al*, 1997) and details have been described earlier (Abts *et al*, 2000). Primers were obtained from GenHunter (RNAimage™, Kit 4, Cat. No. G504). Briefly 1 μg each of total RNA prepared from lesional psoriatic and nonlesional skin specimens was split in three aliquots and reverse transcribed in separate reactions by 200 U Superscript II reverse transcriptase (Gibco BRL) using three HindIII-oligo-dT, one-base-anchored primers modified at their 3′ ends to A, G, and C (H-T_{11}N), and 20 μm of each dNTP according to Liang and Pardee (1992). Display PCR amplification of 2 μl of the cDNAs was carried out between the same anchored primer (each 0.2 μm) and one of 13′mer primers with arbitrary sequence including an HindIII restriction site in a total volume of 20 μl containing 2 U Taq polymerase and 0.4 μl [^{32}p]-α-dCTP (4 μCi). The reactions were hot-started and cycled for 1 min at 95°C, 1 min at 41°C, and 2 min at 72°C in the first PCR cycle, followed by 29 cycles of 50 s at 94°C, 1 min at 42°C (+ temperature increment of 0.1°C per cycle), and 1 min at 72°C. H-AP25 (GenHunter, RNAimage™, Kit 4, Cat. No. G504) was used as an upstream arbitrary primer. Radio-labeled PCR products corresponding to lesional and nonlesional skin were loaded on a denaturing 6% polyacrylamide gel. The gels were run until the xylene cyanol dye reached the bottom, and then were blotted onto Whatman 3M filter paper and dried. Gels were exposed to X-ray film at −70°C for 12 h.

Reamplification After autoradiography, bands of at least 150 bp showing different strength in corresponding lanes were cut out from the dried gel and eluted in 100 μl H_2O. The cDNA was ethanol–glycogen precipitated and the pellet was resuspended in 10 μl H_2O. Using the same primer combination as for the display reaction 4 μl of the eluted material was reamplified using a touch-down protocol in which the annealing temperature was decreased from 50°C to 45°C during the first five cycles and then kept constant at 45°C for 34 additional cycles. An aliquot of the reaction was run on an agarose gel to confirm a reaction product.

Cloning Bluescript KS was digested with EcoRV and incubated with Taq polymerase (Amersham Biosciences, Freiburg, Germany) in the presence of 1 mm TTP to add single base dT residues to the 3′ ends making use of the non-template-dependent activity of the Taq polymerase. Reamplified cDNA fragments were gel-purified using the gel extraction kit (Qiagen, Hilden, Germany) and cloned into the above-described vector via the T/A cloning procedure.

Northern blot hybridization 20 μg of total RNA per lane from multiple psoriasis patients as well as cultured normal keratinocytes and HaCaT cells were electrophoresed and blotted onto Hybond-N+ membrane (Amersham Biosciences). In parallel, a commercially available multitissue Northern blot (Clontech, Heidelberg, Germany) was hybridized. The probe was generated by random-primed labeling of the gel-purified insert. Hybridization was carried out in DIG Easy Hyb solution (Roche, Mannheim, Germany). The autoradiograph signals were quantitated by laser densitometry relative to the band intensities of the corresponding 28S rRNA, which served as loading controls.

Preparation of protein extracts and Western blot analysis The shave biopsies were snap frozen in liquid nitrogen. Under constant supply of liquid nitrogen biopsies were homogenized using a dismembrator (Braun-Melsungen). The powder was immediately transferred into ice-cold lysis buffer containing 25 mm hydroxyethylpiperazine ethanesulfonic acid pH 7.9, 50 mM NaF, 15 mM Triton X-100, 5 mM ethylenediamine tetraacetic acid, 100 mM NaCl, and one tablet protease inhibitor cocktail per 10 ml buffer (Roche).

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lyzed in lysis buffer (see above). The samples were pretreated with ultrasound and cell extracts were centrifuged at 14,000*g* for 20 min at 4°C. The protein concentration of the cellular extracts was determined using the advanced protein assay reagent (TEBU, Frankfurt, Germany). Of the protein extract 20 μg were electrophoresed on 4%–12% NuPage Bis-Tris-Glycin-Gels (Invitrogen, Karlsruhe, Germany) for 2 h at 120 V. Proteins were blotted onto polyvinylidene fluoride membranes (Bio-Rad, Munich, Germany) at 25 V for 90 min using a tank blot system. The membranes were blocked with 5% nonfat dry milk powder in 10 mM Tris–HCl pH 7.5, 150 mM NaCl, and 0.05% Tween 20 (TBST buffer) overnight at 4°C, washed three times with TBST, and incubated with the primary monoclonal mouse antibody against HAX-1 mouse IgG1 (BD Transduction Laboratories), dilution 1:5000 in TBST with 1% nonfat dry milk, overnight at 4°C. The membranes were washed three times with TBST and incubated with the respective secondary antibody (anti mouse IgG, dilution 1:5000, Amersham-Pharmacia, Freiburg, Germany) and visualized using the enhanced chemiluminescence detection system (Amersham-Pharmacia) following the supplier's instructions.

***In situ* hybridization** Cryosections of lesional and nonlesional psoriatic skin on microscope slides were fixed in 4% paraformaldehyde/5% formaldehyde overnight at 4°C. After washing in PBS the slides were prehybridized in precoat solution, containing 5 × sodium citrate/chloride buffer, 50% deionized formamide, 5% blocking reagent (Roche), 0.02% sodium dodecyl sulfate, 0.1% N-lauryl sarcosin, 50 mM Tris–HCl pH 7.4, at 45°C for 1 h. The cRNA probes were synthesized using DIG-UTP and an *in vitro* transcription kit from Roche, following the manufacturer's protocol. As template for the reactions 1 μg of pCRII (Invitrogen) with the HAX-1 fragment, linearized by XhoI, and SP6 polymerase for antisense and BamH I for T7 polymerase for sense orientation were used. 25 ng per 50 μl of the labeled probes were denatured at 94°C for 10 min and added to the prehybridized sections and hybridized overnight at 45°C under a coverslip. Next, the sections were stringency washed with different steps and temperatures. For detection, an alkaline-phosphatase-coupled anti-DIG-antibody and NTB/BCIP as substrate were used.

Sequence analysis Insert-containing plasmids were purified from 10 ml cultures using QIAprep-spin columns (Qiagen, Hilden, Germany), RNAse-treated, and sequenced using the Sequenase Kit from USB (Nebraska) and M13 sequencing primers. The sequences were compared to the EMBL sequence databank entries by FASTA.

Immunohistochemistry Cryosections of psoriatic and healthy skin were fixed in acetone for 20 min at –20°C and then incubated with a polyclonal rabbit anti human HAX-1 antibody (kind gift of Dr. Watanabe, Department of Molecular Immunology, Kyushu University, Japan) for 1 h. After three washing steps in PBS the bound primary antibody was detected by sequential incubation with biotinylated secondary antibodies, peroxidase-coupled avidin, and peroxidase substrate (Vectastain^{R} ABC Kit, Burlingame, CA) as recommended by the manufacturer. To demonstrate the proliferative activity and to appreciate the skin architecture parallel sections were stained with commercially available ready-to-use anti-Ki67 antibody (Dako, Hamburg, Germany) and counterstained with hemalaun.

Transfection and caspase assay Full-length HAX-1 cDNA (accession no. U68566) was amplified with a specific 5′ primer and an oligo dT primer for the 3′ end and cloned into pCRII TA plasmid (Invitrogen). Subsequently, HAX-1 cDNA was subcloned in sense and antisense orientation into pIRES-EGFP (Clontech, Palo Alto, CA).

1×10^6 HaCaT cells were transfected with 1 μg of endotoxin-free plasmid DNA of the HAX–EGFP constructs by electroporation (Amaxa, Cologne, Germany). The transfection efficiency was determined after an additional 16 h incubation by counting the number of green fluorescent cells relative to the total number of cells in three different microscopy fields.

Caspase-3 activity was measured using the ApoAlert CPP32 protease assay kit (Clontech). The transiently transfected cells were exposed to 280 J per m^2 UVB radiation as described earlier (Abts *et al*, 2000) and further incubated for 6 h in new standard culture medium with supplements/serum. Controls were mock-treated. 1.85×10^5 of each of the transfected cell populations as well as nontransfected cells were lyzed. After incubation with the substrate (DEVD-AFC) for 1 h, caspase-3 activity was obtained by measuring the fluorescence emission in a fluorometer (Fluoroscan Ascent Labsystems) (ex 400 nm, em 505 nm). The emission values were corrected for the respective transfection efficiencies: 30% for HAX-1 in sense orientation, 35% for the antisense construct, and 50% for pIRES without insert.

RESULTS

RNA fingerprints were generated from psoriatic lesional and nonlesional skin using DDRT-PCR (**Fig 1**). For each primer combination more than 100 bands were displayed per lane. Use of individual primer combinations resulted in a large number of identical fragments in comparable RNA populations studied. Several products showed different band intensities reflecting different quantities of the corresponding mRNAs. The parallel analysis of four to five related RNA populations helped to discriminate bands that varied randomly between lanes, thus reducing the risk of isolating false positives. Independent DDRT-PCR analysis showed that the band pattern obtained for a given primer combination and cDNA preparation was reproducible (**Fig 1**). The complex pattern revealed several genes that were upregulated or downregulated in lesional psoriatic skin (**Fig 1**). These bands were eluted from the gel, reamplified, and cloned as described above. We further analyzed one particular clone (clone 2) that was differentially expressed in all three psoriasis patients in more detail. The product was generated by amplification using HindIII-oligo-T(11)A and H-AP25 as primers.

The initial verification was performed using slot blot analysis. 1 μg plasmid DNA of the cloned cDNA fragments was immobilized onto nylon membranes under denaturing conditions. Hybridization was performed using radioactively labeled cDNA (1 μg) from lesional and nonlesional skin (data not shown). Although the hybridization confirmed the overexpression of clone 2, clones 8 and 12 were not differentially expressed. Consequently, further experiments were performed with clone 2.

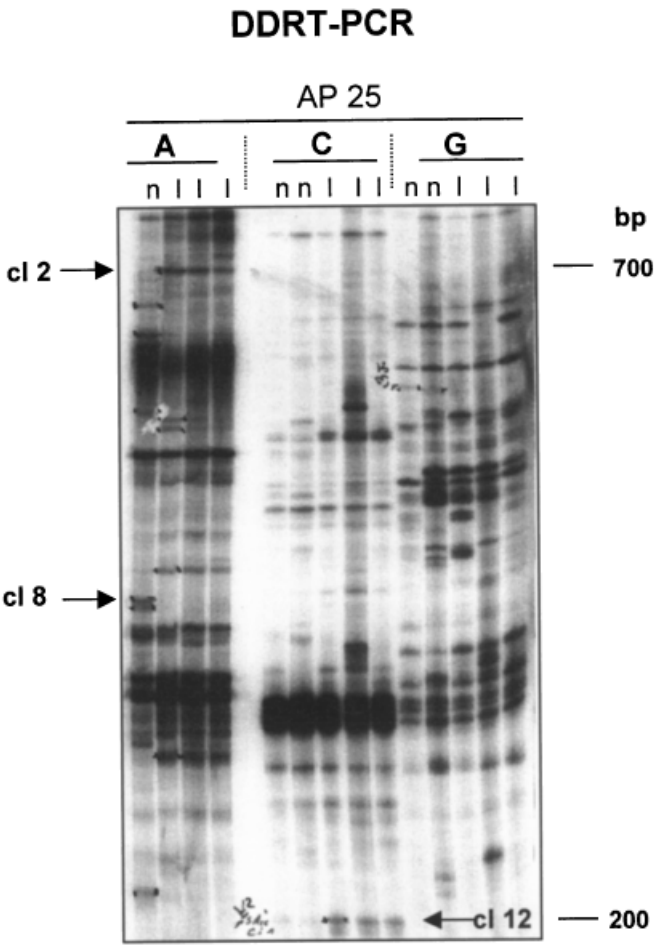

Figure 1. DDRT-PCR with nonlesional (n) and lesional (l) psoriatic skin. The PCR was performed with arbitrary primer (AP) 25 and different one-base-anchored primers A, G, and C. The radiolabeled PCR products were separated on a denaturing 6% polyacrylamide sequencing gel and visualized by exposing to an X-ray film at –70°C for 12 h. The *arrow* shows different cDNAs that are dysregulated in lesional *versus* nonlesional psoriatic skin.

Identification of differential cDNAs by sequence analysis The reamplified and gel-purified cDNAs were cloned and sequenced. Sequence analysis of clone 2 and subsequent FASTA comparison in the EMBL sequence databank revealed 98% homology over a 455 bp stretch of clone 2 to the 3′ translation region of the described 1196 bp HAX-1 coding sequence from lymphocytes (Suzuki *et al*, 1997). Sequence homology of the clone 2 fragment included bases 717–1172 of the entire HAX-1 cDNA.

Northern blot analysis of HAX-1 gene expression To verify the DDRT-PCR results, RNA from psoriasis patients, cultured keratinocytes, and the HaCaT cell line was submitted to Northern blot hybridization with clone 2 and compared to healthy skin (**Fig 2**). In highly proliferative HaCaT cells the expression of HAX-1 mRNA was approximately 13 times stronger than in primary human keratinocytes (**Fig 2**), suggesting a growth advantage by overexpressing HAX-1. We observed a strong upregulation of HAX-1 mRNA of between approximately 2 and 16 times in lesional skin compared to nonlesional and healthy skin (**Fig 2**). The relative transcript levels after densitometric scanning and normalization to the corresponding 28S ribosomal RNA signals are also depicted.

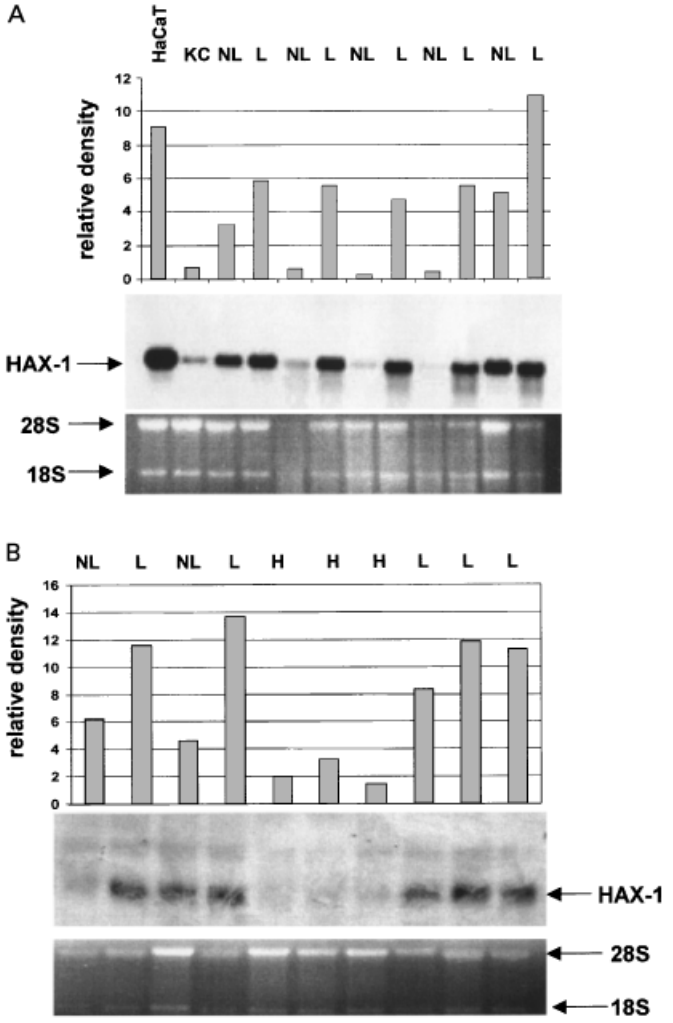

Figure 2. Northern blot analysis of clone 2/HAX-1 mRNA transcription. Clone 2/HAX-1 mRNA levels were elevated in lesional psoriatic skin. (*A*) Northern blot analysis of total RNA from cultured HaCaT cells, normal human keratinocytes (KC), and lesional (L) and nonlesional (NL) psoriatic skin. (*B*) Northern blot analysis of total RNA from nonlesional (NL) and lesional (L) psoriatic skin as well as healthy skin (H) of several volunteers is shown. The signal intensity is compared to 28S RNA.

Using multiple tissue blot, the distribution of HAX-1 mRNA showed a ubiquitous expression pattern with the strongest signals in skeletal and heart muscle (**Fig 3**).

Western blot analysis The Northern blot results were confirmed with the Western blot technique using antibodies against HAX-1 (**Fig 4**). In lesional skin from psoriasis patients HAX-1 was highly overexpressed in contrast to nonlesional and healthy skin (**Fig 4*A***). To assess a potential role of HAX-1 in rapidly proliferating malignant cell lines, HAX-1 protein was analyzed in the highly metastatic (MV3, BLM) and in slowly growing SK-Mel-28 melanoma cell lines and compared to primary melanocytes. HAX-1 was strongly upregulated in MV3 and BLM and in SK-Mel-28 melanoma cell lines (**Fig 4*B***). In immortalized HaCaT cells a strong expression of HAX-1 was observed in comparison to primary keratinocytes (**Fig 4*B***).

The highly metastatic melanoma cell lines MV3 and BLM expressed HAX-1 protein three times more strongly than nontumorigenic HaCaT cells (**Fig 4*B***).

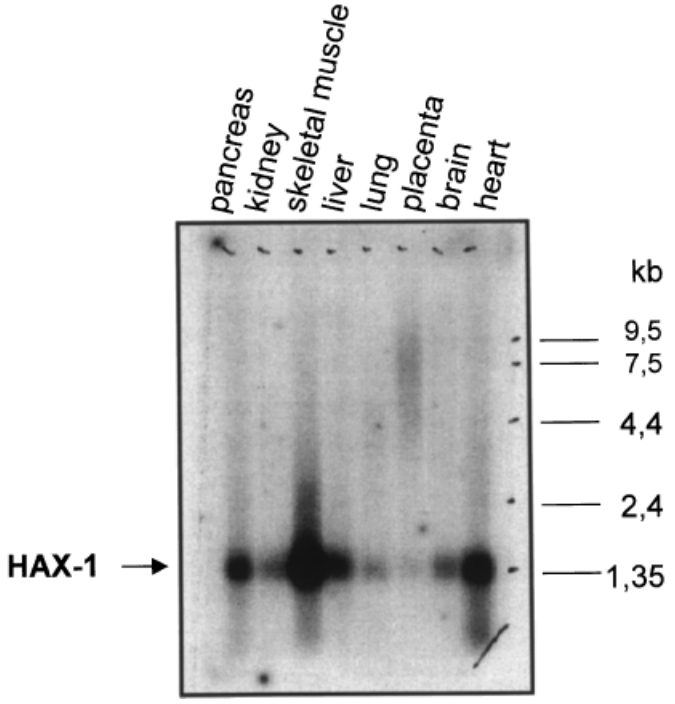

Figure 3. Multiple tissue Northern blot analysis. Metabolically active tissues express the highest levels of clone 2/HAX-1. Autoradiograph of a multiple tissue Northern blot of mRNAs from different tissues probed with the insert of clone 2 and exposed overnight.

In situ* hybridization and immunohistochemistry** Tissue distribution studies of clone 2 mRNA in skin revealed a strong positive signal in all layers of lesional psoriatic skin (**Fig 5*B). The dermis was virtually free of clone 2 hybridization signals in all specimens examined except for some inflammatory cells. The negative control (sense orientation) showed only background staining (**Fig 5*C***). The signal in healthy skin was weak but definitive and also in the basal and spinous layers (**Fig 5*A***).

The antibody used for immunohistochemistry detection of HAX-1 was a kind gift of Dr. Watanabe (Suzuki *et al*, 1997). The HAX-1 protein distribution correlated very well with the localization of the transcripts described above. In healthy skin the strongest staining was located in basal keratinocytes (**Fig 5*D***); however, in suprabasal layers a weak staining was also detected (**Fig 5*D***). In psoriatic skin HAX-1 was distributed as a gradient with the highest levels in basal layers and positive staining in the granular layers (**Fig 5*F*, *H***). The basal keratinocytes of both healthy and psoriatic skin also showed the highest staining of the proliferation-associated nuclear antigen Ki67 (**Fig 5*E*, *G*, *I***). In healthy skin the highest levels of HAX-1 mRNA and protein could be colocalized to Ki67-positive, proliferating keratinocytes in the basal epidermal layer, where cells are still undifferentiated. In suprabasal, progressively differentiating layers HAX-1 expression was markedly reduced but still detectable. The outermost epidermal layers were negative for HAX-1. In contrast with healthy skin, HAX-1 was strongly expressed in basal, spinous, and granular layers in lesional psoriatic skin, where the terminal differentiation of keratinocytes is disturbed. In lesional psoriatic skin Ki67 was expressed in basal and in a few suprabasal epidermal layers. The parallel Ki67 staining showed that HAX-1 expression was distinct.

Caspase-3 assay A clear indication of an antiapoptotic function of HAX-1 in epidermis results from our transfection/irradiation

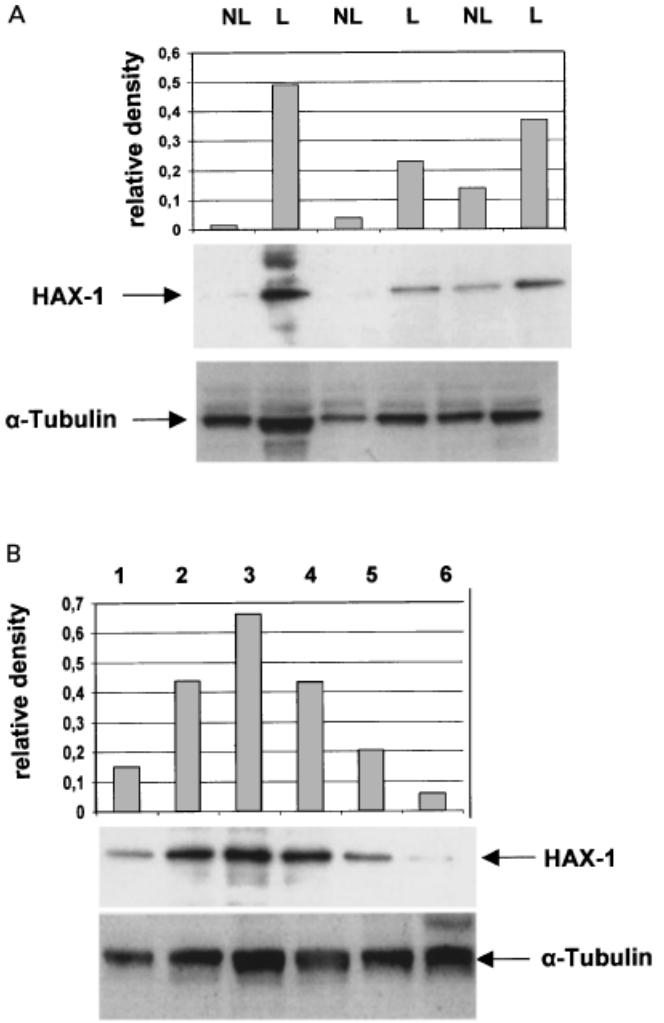

Figure 4. Western blot analysis of clone 2/HAX-1 expression. To confirm protein expression Western blot analysis was performed on protein extracts of nonlesional (nl) *versus* lesional (l) psoriatic skin of three different patients (*A*). (*B*) Expression of HAX-1 primary keratinocytes *versus* HaCaT cells, and in different melanoma cell lines *versus* primary melanocytes, respectively: *1*, primary melanocytes; *2*, MV3; *3*, BLM; *4*, SK-Mel-28; *5*, HaCaT; *6*, primary keratinocytes. The signal intensity is compared to α-tubulin.

experiments. The induction of apoptosis by UV light was described earlier (Schwarz *et al*, 1995). UV irradiation caused an approximately 10-fold induction of caspase-3 activity in HaCaT cells (**Fig 6**). Enzyme activity could be further increased to a level of 20-fold compared to unirradiated cells when a plasmid construct expressing HAX-1 antisense mRNA was transfected into the cells. Transfection of both HAX-1 in sense orientation or a control vector without the insert led to an increase in caspase activity in irradiated cells of up to the level of untransfected cells.

DISCUSSION

Psoriasis is a chronic inflammatory disease characterized by increased proliferation and altered differentiation of keratinocytes, in the context of vascular alterations and epidermal infiltration of activated T lymphocytes. In order to characterize genes differently expressed in psoriasis, gene expression within psoriatic lesions was compared to healthy skin using DDRT-PCR. Compared to cDNA arrays, the DDRT-PCR technique allows identification and isolation of a broad variety of differently regulated genes without preselection and independent of any existing gene sequence. By using this technique, the different expression of a number of clones was verified by slot blot analysis to prevent further analysis of false positives, a major drawback of the DDRT-PCR technique (Liang and Pardee, 1995). As a result of this, the differential expression of clone 2 was further substantiated on the transcription and protein level using Northern and Western blot analysis, respectively.

Sequence analysis in the gene bank found clone 2 to be identical to the cDNA sequence encoding for the HS1-binding protein HAX 1. This 35 kDa protein was initially identified in lymphatic cells and shown to influence the B cell receptor (BCR) signaling pathway by interaction with HS1, potentially counteracting the cell-death function of HS-1 in lymphocytes (Fukuda *et al*, 1995; Taniuchi *et al*, 1995; Suzuki *et al*, 1997).

As we found HAX-1 to be upregulated in psoriatic lesions, a functional relevance in human skin can be assumed. Subsequently, Northern and Western blot analysis confirmed the rather weak HAX-1 expression in healthy and nonlesional skin compared to hyperproliferative psoriatic lesions as initially shown by DDRT-PCR analysis. The genomic localization within the epidermal differentiation complex on chromosome 1q21 suggests a regulating function during the epidermal maturation process (Marenholz *et al*, 2001). Analysis of the HAX-1 amino acid sequence revealed similarity to the functionally important domains BH1 and BH2 of the Bcl-2 subfamily of proteins, which counteract apoptosis (Kroemer, 1997). These proteins are, as HAX-1, mainly located in mitochondria, nuclear envelope, and endoplasmatic reticulum (Suzuki *et al*, 1997). According to the structural and locational homologies to antiapoptotic proteins, the authors speculated that HAX-1 promotes cell survival by inhibiting apoptosis.

Indeed, tissues with high numbers of mitochondria, indicative for high energy metabolism, such as brain, heart, skeletal muscles, and pancreas, show high HAX-1 levels (**Fig 5**). Assuming an antiapoptotic activity, increased HAX-1 expression could be assumed to protect from cell death due to respiratory stress in tissues with high energy metabolism. In the opposite case, placental tissue presenting a rather low level metabolism with a high turnover rate shows only low HAX-1 levels.

Further supportive findings for involvement of HAX-1 in apoptotic processes reveal sequence homology of HAX-1 to Nip3, an adenovirus E1B interacting protein that itself has been shown to interact with several antiapoptotic viral and cellular proteins, including Bcl-2 and BHRF-1 (Sharp *et al*, 2002). Consistent with the regulatory function of Nip3 in apoptosis, a T lymphoma cell line acquired resistance to apoptosis following various stimuli, including Fas treatment, γ-irradiation, and serum deprivation, when HAX-1 was overexpressed (Suzuki *et al*, 1997). Moreover, several proteins have recently been identified to interact with HAX-1, such as the Epstein–Barr virus nuclear antigen 5 and latent protein, which may be involved in immortalization of B lymphocytes (Kawaguchi *et al*, 2000; Dufva *et al*, 2001), or the K15 protein of Kaposi's sarcoma-associated herpesvirus, which may regulate viral latency or promote growth of the infected cells (Hengge *et al*, 2002; Sharp *et al*, 2002). The expression of HAX-1 in several cell lines in contrast to their primary counterparts suggests a broader role in immortalization.

Beside an increased proliferation rate a diminished susceptibility to apoptosis is assumed heading to epidermal hyperproliferation in psoriasis (Collins and Lopez Rivas, 1993; Laporte *et al*, 2000). Indeed, keratinocytes isolated from psoriatic lesions showed an increased expression of the antiapoptotic Bcl-x that can be further induced by interferon-γ, one of the key cytokines in the pathogenesis of psoriasis (Nickoloff, 1999). The induction of antiapoptotic proteins together with antiproliferative effects of interferon-γ in psoriasis indicate that proliferation and antiapoptosis are independent.

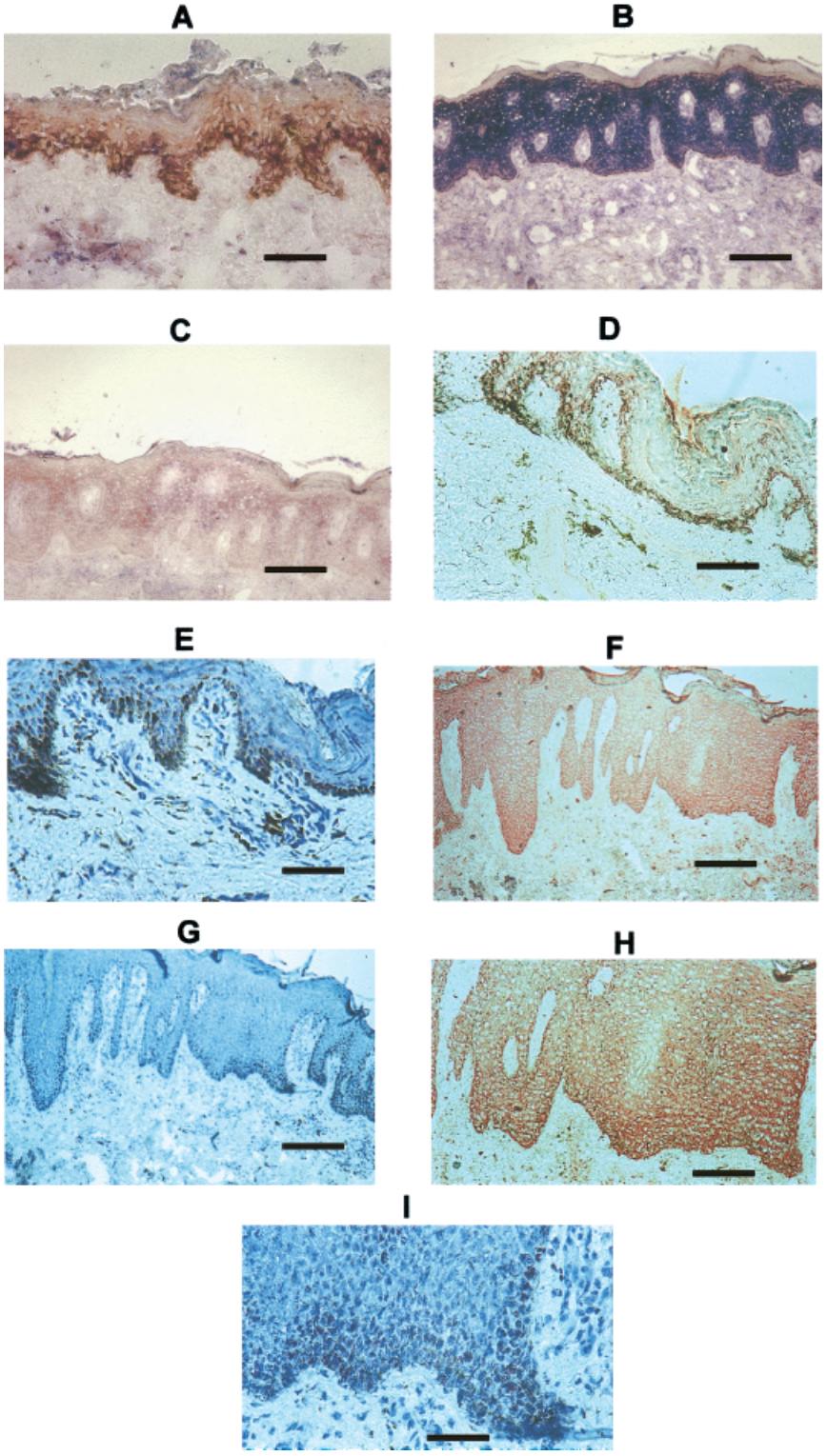

Figure 5. *In situ* hybridization and immunohistochemistry. Clone 2/HAX-1 mRNA and HAX-1 protein localization in normal *versus* lesional psoriatic skin. (*A*)–(*C*) *In situ* hybridizations of cryosections of healthy (*A*) and lesional psoriatic skin (*B*) with antisense cRNA, generated from clone 2. (*C*) Negative control where a parallel section of lesional skin was hybridized to sense cRNA of clone 2. (*D*)–(*I*) Immunohistochemical staining for HAX-1. Healthy skin (*D*) with HAX-1 antibody and (*E*) with Ki67 antibody. Lesional psoriatic skin (*F*) with HAX-1 antibody and (*G*) with Ki67 antibody. (*H*)–(*I*) Close-up view of (*F*) and (*G*), respectively. *Scale bars*: (*A*), (*B*), (*C*), (*F*), (*G*) 200 μm; (*D*), (*E*) 100 μm; (*H*), (*I*) 50 μm.

In order to find further evidence for the role of HAX-1 in antiapoptotic processes, UVB-induced apoptosis was analyzed in HaCaT keratinocytes overexpressing HAX-1 mRNA or its antisense. According to previous findings, UVB irradiation of untransfected cells caused a strong increase of caspase-3 activity, indicative of terminal activation of the apoptotic cascade (Schwarz *et al*, 1995). In contrast, specific antisense mRNA inhibition led to a marked increase in caspase-3 mediated apoptosis, strongly suggesting an antiapoptotic role for HAX-1.

The data presented here have shown that DDRT-PCR is a useful technique for the isolation of differently expressed genes in psoriasis. These initial PCR results were supported by further expressional analysis and the overexpressed clone 2 could be identified as HAX-1. Future experiments will elucidate the exact role of HAX-1 in apoptotic processes. Detailed analysis of the expression of HAX-1 in conjunction with the imbalance of pro- and anti-inflammatory cytokines in psoriatic lesions is of great interest, possibly facilitating the development of new anti-psoriatic drugs.

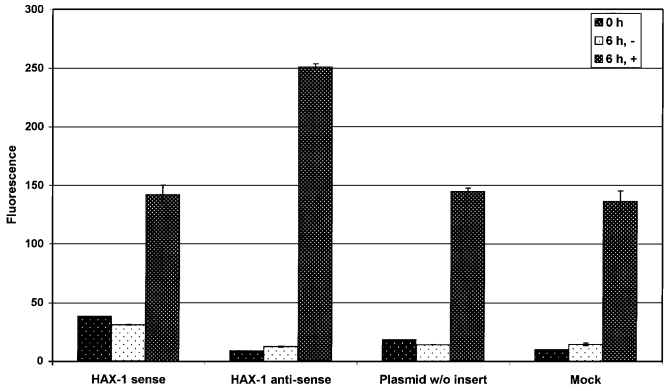

Figure 6. ApoAlert caspase-3 assay in transiently transfected HaCaT cells after irradiation with 280 J per m^2 UVB. HAX-1 antisense RNA enhances UVB-induced apoptosis. HaCaT cells were transfected with plasmid constructs expressing HAX-1 sense or antisense mRNA. The negative controls consisted of vector without insert and mock-transfected cells (HaCaT). Caspase-3 activity was determined fluorometrically. A representative experiment is shown.

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